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Statistical optimization of *Aspergillus flavus* cell bound saponin hydrolase production by Taguchi DOE methodology

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ABSTRACT

Soyasapogenols have greater in vitro cellular anticancer activity compared to their corresponding glycosides. Soyasapogenol B (SB) has many medicinal applications due to hepatoprotective, antivirus, anti-inflammatory, antimutagenic and anticancer activities. SB can be produced by enzymatic hydrolysis of soyasaponins (SS) using microorganisms having saponin hydrolase (SH) activity. SH production by Aspergillus flavus has been modeled and optimized using Taguchi's orthogonal methodology. L_{30} array with four factors at three different levels was constructed using Design-Expert software. The ANOVA analysis revealed maximum F ratio of 43.71 in case of SS concentration and consequently proved to be the most influential factor with a contribution percentage of 31.65%. The optimum conditions for maximum production of SH activity and SB yield were; 1% glucose, 1.3% SS, medium pH 7 and 4 days incubation period. The produced model has been validated and the results showed 79.06% validation confidence. An enhancement of 137.3 % in SH production compared to the original production medium was detected and SB yield was significantly improved after application of Taguchi method. Consequently, Taguchi method was proved to be effective in optimizing the culture conditions of SH production.

Keywords: Saponin hydrolase. Soyasapogenol B. *Aspergillus flavus*. Taguchi DOE methodology.

INTRODUCTION

Soyasaponins (SS) are triterpenoid glycosides present in legume seeds. Their basic structure is an oleanene- type triterpenoid aglycone and sugar residues attached to it [1]. SS are divided into Groups A, B and E in plants according to their respective aglycones, soyasapogenol A, soyasapogenol B (SB) and soyasapogenol E [2]. Group B SS have one sugar chain attached to the C-3 position of SB [1].

Soyasaponins have been reported to have diverse biological actions including plasma cholesterol lowering, antifungal, antiparasitic, anti-viral activities, antioxidant activities and antitumor activities [3,4,5,6,7,8]. However, soyasapogenols have greater *in vitro* cellular anticancer activity compared to the corresponding glycosides [1]. SB is known to have hepatoprotective, antivirus, anti-inflammatory, antimutagenic and anticancer activities [9,10,11,12]. Soyasapogenols are produced by acid hydrolysis of saponins [11]. However, enzymatic hydrolysis is a well-known environmental friendly process compared with chemical hydrolysis due to its high selectivity and mild reaction conditions [13]. Based on this evidence, SB could be produced from SS using microorganisms having saponin hydrolase (SH) activity. SH was isolated from different fungi such as *Aspergillus oryzae* PF1224 and *Neocosmospora vasinfecta* var. *vasinfecta* PF1225 [10,14].

Optimization of SH production conditions was previously studied by varying one factor at a time approach [15]. However, this strategy is time consuming and laborious for a large number of variables identification and tends to

overlook interactions among variables. Alternatively, statistical design of experiments (DOE) can be used. It is a collection of mathematical and statistical analysis that is useful for determining the factors that influence the response and/or their optimum levels [16]. Taguchi DOE method is an orthogonal array design which is fast way of optimization, reduces experimental errors, and optimization could be achieved in an economical way [17]. For the best of our knowledge, there have been no reports investigating SH production using factorial experimental designs.
In the course of our study of SB production from SS using whole cells producing SH, *Aspergillus flavus*, a fungus isolated from peanut pods, was screened on the basis of its ability to produce SH enzyme [15]. The effects of SS concentration, glucose addition, pH and incubation period on SH production by *A. flavus* cells [15] were previously studied in the production medium using the 'one-variable-at-a-time' method. Here, SH production from *A. flavus* was optimized by Taguchi orthogonal array method which is the first to be reported till now.

MATERIALS AND METHODS

Microorganism and cultivation
A. flavus, a fungus isolated from peanut pods, was maintained on potato dextrose agar (PDA-Difco). SH production was carried out in production medium containing (g/l) 10 SS, 40 malt extract, 20 yeast extract, 2 KH_2PO_4, 2 $(NH_4)_2SO_4$, 0.3 $MgSO_4 \cdot 7H_2O$ and 0.3 $CaCl_2 \cdot 2H_2O$ with a pH adjusted to 7.0. *A. flavus* was cultured in Erlenmeyer flasks (250 ml) containing 100 ml of production medium and incubated for 72h on a rotary shaker at 150 rpm and 30±2 ^oC.

Measurement of SH activity
Cells SH activities were measured as follows; 1g wet cells was added to 5ml of 2% SS suspended in 0.2M acetate (pH 5.5) and the mixture was allowed to react at 45°C for 1h. Reaction products were extracted twice with 5ml of ethyl acetate. The quantity of SB in the sample was analyzed by high-pressure liquid chromatography (HPLC). One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmole of aglycone (SB) *per* hour from the substrate. Cell based specific activity can be defined as µmole of aglycone *per* hour *per* g dry weight cells.

SB analytical methods
Thin layer chromatography (TLC) was carried out on pre-coated silica gel plate (Merck, silica gel 60F-254). The plate was chromatographed for SB with a solvent system of benzene: ethyl acetate: acetic acid (12:4:0.5, v/v/v). SB having an Rf value of 0.35 was detected on TLC plates by acid charring (10% H_2SO_4, 120◦C, 10 min).

HPLC was performed with Waters Alliance HPLC System (Model NO.E2695 XE Separations Module, Austria) under the following conditions: column, Sun Fire Prep C18 (5µm, 10x150mm); column temperature, 40°C; mobile phase, acetonitrile-methanol-water (50: 15: 35); flow rate, 1 ml/min; and UV detector operating 200 nm. 100 µl of ethyl acetate containing reaction products was diluted with 900 µl of the mobile phase. 10 µl of this dilution was analyzed by HPLC, and the quantity of SB in the sample was determined by comparison with authentic SB [18]. The SB yield (%) in the reaction mixture was calculated using Eq. 1.

$$\text{SB yield (\%)} = \frac{[\text{soyasapogenol B weight} / \text{soyasapogenol B MW}] \times 100}{[\text{soyasaponin I weight} / \text{soyasaponin I MW}]} \quad (1)$$

Where MW is the molecular weight; soyasaponin I represents SS.

Experimental design for optimization of *A. flavus* SH production conditions

Taguchi DOE model was used to study the effect and interactions between glucose concentration (A) in the range between 0 and 2%, SS concentration (B) in the range between 1 and 2%, pH (C) in the range between 7 and 9 and incubation period (D) between 2 and 4 days (Table 1) for maximum production of SH and SB yield by *A. flavus.* L30 orthogonal array was constructed based on the four selected factors at 3 different levels. The levels of the studied factors and the L30 orthogonal array are shown in Table 2. Experimental designs were performed using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, ver 7.0.0). A total of 30 experiments were employed in Taguchi to estimate curvature and interaction effects of selected variables. At the end of the incubation period of each run, the SH activity was assayed in whole cells as well as in cultural filtrate. Finally, the significance of the obtained model was checked by t-test (calculated *p*-value) and goodness of fit by multiple correlations as well as determination coefficients.

Statistical Analysis
Analysis of variance (ANOVA) was used to estimate the statistical parameters for optimization of culture conditions. A probability value of p value <0.05 was used as the criterion for statistical significance.

Validation of Taguchi design

The factors affecting SH activity and SB yield were numerically optimized for theoretical maximization of production. The optimum conditions were, practically, tested and compared with the theoretical results to validate the production model. After optimization and validation of the conditions, three-dimensional surface figures were plotted in order to illustrate the relationship between SB yield and the experimental variables used.

RESULTS AND DISCUSSION

Many factors are thought to control the enzyme secretion including; the nutritional, physiological, and biochemical nature of the microorganism employed, and even on the strain of the microorganism [19]. Many fungal strains have been marked as SH producing microorganisms. These strains include *Aspergillus parasiticus* [20], *Aspergillus terreus* [21], *Aspergillus oryzae* KO-2 [22] and *Neocosmospora vasinfecta var. vasinfecta PF1225* [18]. Moreover, Kudou *et al.* [23] reported 26 strains of *Aspergillus* with soybean saponin (SS) hydrolyzing activity. Obviously, *Aspergillus* species have attracted much attention, because they were the most significant producers.

Table 1: Culture conditions factors and assigned levels selected for optimization

Factor	Unit	Low level	Intermediate level	High level
Glucose concentration	%	0	1	2
SS concentration	%	1	1.5	2
Medium pH		7	8	9
Incubation period	days	2	3	4

Table 2: L_{30} Taguchi orthogonal array of designed experiments

Run	Glucose concentration	SS concentration	Medium pH	Incubation period
1	1	2	7	3
2	2	1.5	7	4
3	1	1	8	4
4	1	1.5	9	2
5	0	2	9	4
6	0	1.5	8	3
7	2	1	9	3
8	0	1	7	2
9	2	2	8	2
10	1	2	7	3
11	2	1.5	7	4
12	1	1	8	4
13	1	1.5	9	2
14	0	2	9	4
15	0	1.5	8	3
16	2	1	9	3
17	0	1	7	2
18	2	2	8	2
19	1	2	7	3
20	2	1.5	7	4
21	1	1	8	4
22	1	1.5	9	2
23	0	2	9	4
24	0	1.5	8	3
25	2	1	9	3
26	0	1	7	2
27	2	2	8	2
28	1	1.5	8	3
29	1	1.5	8	3
30	1	1.5	8	3

Analysis of the relationships among a number of parameters that affect the overall process can be achieved using statistical experimental design. This procedure can be very effective for identification of the optimum operational conditions for enzyme production. *A. flavus* SH was proved to be mainly cell bound enzyme, which contributed more than 60% of the total enzymatic activity in the production medium [15]. Therefore, optimization of enzyme production by whole cells resulting in production of cells has a maximum enzyme activity to be used for SS conversion to SB. Taguchi orthogonal array (OA) design has been proved as a promising way for the optimization of microbial enzymes production over conventional methods [24]. This method could involve a mixture of physical and nutritional factors in the concise fractional factorial designs [19]. Glucose concentration, SS concentration, medium pH and incubation period were selected for medium optimization for enhanced SH production because they had significant impact on SH production as screened in our earlier findings [15]. The SH activity and SB yield of L_{30}

orthogonal array are shown in Table 3. A significant variation in enzyme production ranging from 18.87 to 63.33 U/g (equivalent to SB yield from 8.50 to 28.54%, respectively; Table 3) was recorded. Moreover, the correlation between the actual and predicted response for SB yield was shown in Fig. 1. A good convergence between actual (experimental) and predicted results can be noticed.

Table 3: SH activities and corresponding SB yields of Taguchi design experiments

Run order	SH activity (U/g)	SB yield (%)		
		Actual	Predicted	Residual
1	48.57642	15.21269	13.59727	1.615419
2	45.92949	10.13482	11.37224	-1.23742
3	45.57893	0	-0.21087	0.210873
4	26.47479	12.04635	10.25597	1.790379
5	36.2185	18.71341	19.09103	-0.37762
6	43.0241	9.673084	18.00197	-8.32889
7	49.42732	9.408207	4.703217	4.70499
8	35.5755	2.142189	1.810194	0.331995
9	18.86616	14.46842	12.62386	1.844557
10	54.01481	12.48243	13.59727	-1.11484
11	45.44434	12.19074	11.37224	0.818504
12	42.84463	0	-0.21087	0.210873
13	24.88211	8.27677	10.25597	-1.9792
14	44.90929	19.65381	19.09103	0.562776
15	43.54612	24.19938	18.00197	6.197409
16	60.0313	4.697198	4.703217	-0.00602
17	42.31444	0.849827	1.810194	-0.96037
18	21.9289	13.88361	12.62386	1.259747
19	54.19957	13.71657	13.59727	0.119299
20	48.34375	11.7869	11.37224	0.414664
21	47.66159	0	-0.21087	0.210873
22	21.05509	11.06467	10.25597	0.808699
23	48.50182	18.27751	19.09103	-0.81352
24	44.48226	21.39019	18.00197	3.388219
25	63.32523	0	4.703217	-4.70322
26	0	25.29	20.13	5.16
27	27.99281	9.528051	12.62386	-3.09581
28	56.90717	12.85758	12.91503	-0.05745
29	54.91447	13.2976	12.91503	0.382572
30	54.07865	10.71753	12.91503	-2.1975

Table 4: Analysis of variance (ANOVA)

Source	Sum of Squares	Mean Square	F-Value	p-value Prob > F*	Percentage, P (%)
Model	1047.22	149.60	15.76	< 0.0001	
A-Glucose Conc.	48.20	48.20	5.08	0.0351	3.676302
B-SS Conc.	414.95	414.95	43.71	< 0.0001	31.649
C-Medium pH	24.47	24.47	2.58	0.1233	1.866372
D-Incubation period	69.69	69.69	7.34	0.0131	5.315384
AC	58.42	58.42	6.15	0.0217	4.4558
A^2	77.31	77.31	8.14	0.0095	5.896575
B^2	219.32	219.32	23.10	< 0.0001	16.72794
Residual	199.37	9.49			
Lack of Fit	2.41	1.21	0.12	0.8907	0.183815
Pure Error	196.96	10.37			15.0225
Cor Total	1246.59				

**: Values of "Prob > F" less than 0.05 indicate model terms are significant.*

Table 5: Validation of the Taguchi model

Test type	Glucose Conc. (%)	SS Conc. (%)	Medium pH	Incubation period (d)	SB Yield (%)		Experimental SH (U/g)
					Mathematical	Experimental	
Test validation	2	1.7	8	3	31.97272	40.72618	77.1213
	0	1.8	7	3	35.41088	40.01351	75.77175
	1	1.3	7	4	35.63257	48.61781	92.06532
	2	1.3	9	4	36.83362	48.49318	91.82931
Control	0	1	7	3		35.31727	66.8787

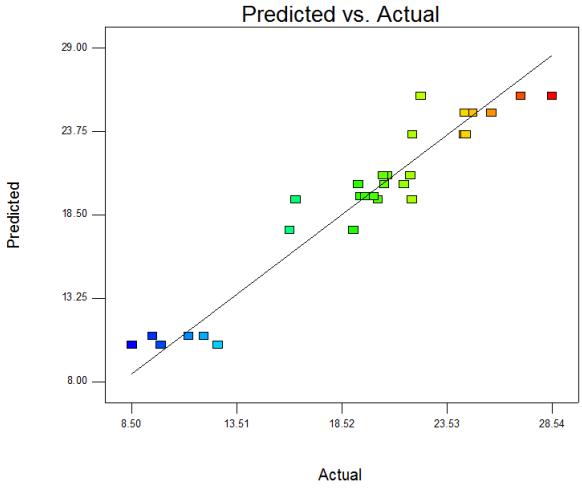

Fig. 1: Correlation between the actual and predicted response for SB yield

The significant levels of selected factors and their relative contribution to SH production were determined using the analysis of variance (ANOVA) of Taguchi model (Table 4). Statistical analysis of SB yield data using above experimental designs (Table 3) showed that the model is significant, since the model *F*-value is 15.76. From the calculated ratios (*F*), the factors; A, B, D, AC, A^2 and B^2 are significant model terms at 95% confidence limit. This revealed that almost all the factors considered in the design had significant effects except medium pH (C) had no significant effect on SB yield at the individual level. Although non significant, medium pH had a significant effect on SB yield as a multiplication product with glucose. While, SS has the maximum *F*-ratio of 43.71, the minimum *F*-ratio of 2.58 was observed in case of medium pH. The model lack of fit value of 0.12 implies that the model is not significant relative to the pure error.

The analysis of data by ANOVA also revealed about the percentage contribution of selected factors (Table 4) on enzyme production. Among all selected factors, the maximum contribution was inhereted to SS (31.649 %) on SB yield and overall SH production process. Consequently, SS is proved to be a critial factor for stimulation of SH production by *A. flavus*. It was reported that SH enzyme was induced by the presence of SS in the fermentation medium [23]. Incubation period, glucose and medium pH showed lower contribution percentages (5.32, 3.68 and 1.87%, respectively) compared with SS. Medium pH showed the least percentage contribution at the individual level. This revealed that SH could be produced efficienlty under the tested pH range (7-9).

The data analysis showed that the model has R^2 value (multiple correlation coefficients) of 0. 8401, which revealed that the model could explain 84.01 % variation in the response.The predicted-R^2 of 0.7119 was in reasonable agreement with the adjusted-R^2 of 0.7868. All three factors were positive and close to each other, indicating the good statistical model [25]. Adequate precision ratio of 11.927 indicates an adequate signal. Accordingly, this model can be used to navigate the design space.

$$\text{SB Yield (\%)} = -52.50278 - 55.57322*A + 109.50699*B - 4.67574*C + 3.87081*D + 5.88750*A*C + 3.38630*A^2 - 30.20543*B^2 \quad (2)$$

The regression equation (Eq. 2) for SB yield in terms of actual factors could be applied on SH production, as both parameters (SB yield and SH activity) based on the determined SB concentration in the enzyme reaction mixture. It showed that the increase in SS concentration, incubation period, squared glucose concentration and multipied glucose concentration and medium pH have positive effects on SH production. A unit increase of squared glucose concentration, incubation period, the product of glucose concentration and medium pH multiplication and SS concentration results in 3.3863, 3.87081, 5.8875 and 109.5 increase in SB yield, respectively. This result reflects the dependance of SH productivity on SS as an inducer. In contrast, the increase in glucose concentration, medium pH and squared soyasaponin concentration have negative effects on SH production. A unit increase of glucose concentration, medium pH and squared SS concentration results in 55.57322, 4.67574 and 30.20543 decrease in SB yield, respectively.

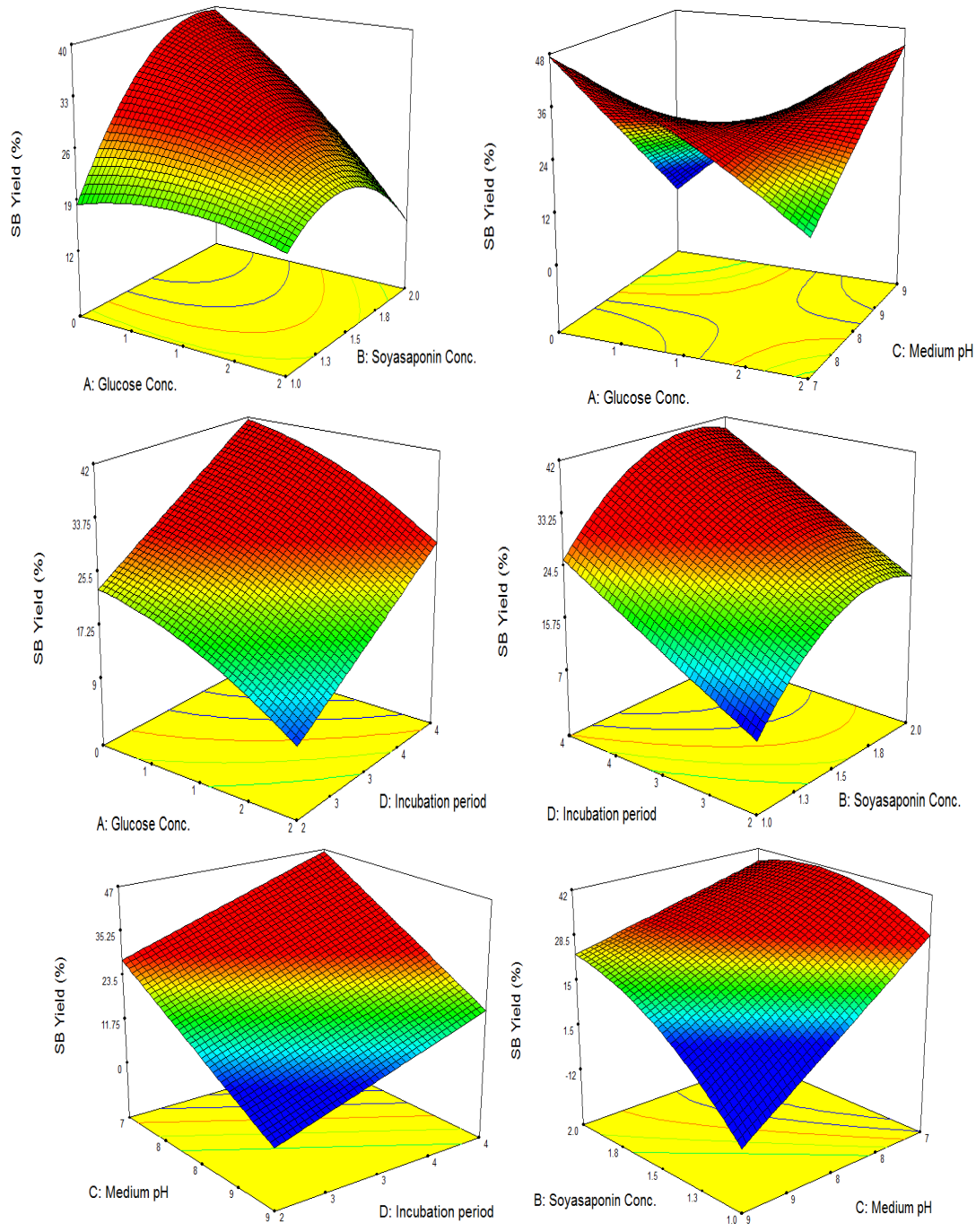

Fig. 2: 3D plots for interactions between different factors and the effect on SB yield

Validation of Taguchi design

To validate the statistical model and regression equation, further experiments were performed using four different proposed optimized culture conditions as represented in Table 5. The mathematical along with the experimental results of the optimization of the different factors affecting SH production were determined. The optimum culture conditions for maximum production of SH were: 1% glucose, 1.3% SS, pH 7 and 4 days incubation period. However, in a previous study using the 'one-variable-at-a-time' method *A. flavus* cells with maximum SH activity was obtained using production medium supplemented by 2% SS, as inducer for enzyme production, adjusted at pH 9 and incubated at 30°C for 2 days [15]. The validation resulted in SB yield of 48.62% (expected response 35.63%), consequently the model is valid with 79.06 % confidence. While at the beginning of the experiments the SB yield was about 35.32%, after using Taguchi optimization process it was raised to 48.62% (37.66 % increase). Also, the enzyme produced was increased from 66.87U/g to 92.07 U/g, indicating an increase of about 137.3 % in production of SH compared with the original production medium. Three dimensional plots (Fig. 2) of the different factors show factor-factor interaction and the effect of these factors on SB yield. From these figures, it was noticed that SB yield

decreases with the increase in glucose concentration. In contrary, SB yield increases with the increase in SS concentration and vice versa. These features can be explained as the presence of glucose, as a simple and easy to digest carbon source, is favored by the microorganism over SS and therefore inhibits SH. Whereas, the presence of SS induce production of SH in the absence or at low concentrations of glucose. The effect of variation in pH revealed that SH production increases at neutral conditions compared with basic ones, except at high concentration of glucose at which the conditions adverse. This behavior can be explained based on acid production as a result of glucose fermentation which neutralizes the pH (as an optimum pH condition) if the initial pH was alkaline and make it more acidic if it was neutral. On the other hand, the behavior of the incubation period on SH production was rational as it increases with time increase.

CONCLUSION

The present study is considered as the first report investigating the statistical optimization and validation of SH production conditions by *A. flavus*. Taguchi DOE array (L_{30}) with four factors at three levels was applied successfully. Among the four factors, SS individually was proved to be the most significant factor. After numerical optimization and validation, the SH activity was increased with about 137.3 % compared with the original production medium. Moreover, SS conversion to SB was significantly improved (37.66% increase) under the optimized conditions.

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